

IMMUNOFLUORESCENT STAINING FOR RESPIRATORY VIRUS DETECTION: INFLUENZA A, INFLUENZA B AND RESPIRATORY SYNCYTIAL VIRUS

A. Introduction

Influenza A, Influenza B and RSV are responsible for serious respiratory infections. Immunofluorescent staining allows for rapid diagnosis of these agents directly from upper respiratory tract specimens.

B. Principle

Influenza A and Influenza B viruses are detected by an indirect immunofluorescence technique. Monoclonal antibodies specific to each virus, bind to antigen expressed in the cytoplasm of infected cells. Fluoresceine labeled mouse anti-IgG in the conjugate binds to these antibodies and the cells exhibit fluorescence upon microscopy.

RSV is detected by a direct immunofluorescence technique. Fluoresceine labeled monoclonal antibodies bind directly to antigen expressed in the cytoplasm of infected cells. These cells then exhibit fluorescence upon microscopy.

C. Specimen Collection

1. Specimen collection preparation.
 - a. Assemble in the lab the following collection supplies.
 - For Nasopharyngeal Swabs (NPS).
 - i. 2 swabs: cotton, dacron, or nylon swab; wood or plastic shaft.
 - ii. A glass specimen slide (immunoslides with 8 wells).
 - iii. A transport vial with screw cap.
 - iv. Influenza Surveillance Form (see attachment 1).
2. Nasopharyngeal Swab (NPS).
 - a. Label the provided specimen slide and transport tube with patient name, patient ID number and collection date.
 - b. Use 2 swabs for collection: a cotton, dacron, or nylon swab.
 - c. Insert the first swab into one nostril parallel to the palate.
 - d. Rotate the swab gently and advance until resistance is felt.
 - e. Press swab tip on the mucosal surface of the interior nostril.
 - f. Leave in place for a few seconds then slowly withdraw with a rotating motion.
 - g. Smear the swab on the labeled specimen slide, ensure that nasopharyngeal material is visible on the slide and is smeared on all 8 wells of the slide. Allow the slide to air dry completely.
 - h. Insert the second swab into the other nostril and repeat steps d to f above.
 - i. After withdrawing the second swab, break it off into the labeled transport vial.
 - j. Complete surveillance form and deliver specimen and form to the laboratory as soon as possible.
3. Nasopharyngeal Aspirate (NPA).
 - a. Extend the patients head back as far as possible.
 - b. Apply 2.5 ml of sterile normal saline into one nostril with a syringe.
 - c. Collect the nasal wash by placing a clean specimen container directly under the nose, tilt the head forward and allow the fluid to run into the container.
 - d. Alternatively, the fluid may be aspirated with a syringe and sterile tubing and then transferred into a clean container.
 - e. Repeat the steps with the other nostril.
 - f. Complete the surveillance form and deliver the NPA specimen to the lab as soon as possible for testing.

D. Specimen Criteria

1. Acceptable Specimens.
 - a. Nasopharyngeal swabs.
 - i. Cotton, dacron, or nylon swabs; wood or plastic shaft.
 - ii. To be smeared immediately onto a suitable specimen slide and air dried.
 - iii. Specimen slides must be delivered to the lab within 1 hour after collection.
 - iv. Two specimens must be delivered to the lab: one specimen slide smeared with the NPS material and one swab broken off into a labeled transport vial.
 - b. Nasopharyngeal aspirate.
 - i. To be collected in a clean, leak-proof container.
 - ii. Specimen must be delivered to the lab within 1 hour after collection.
 - iii. If longer than 1 hour, store specimen at refrigerated temperature prior to delivery to lab.
 - c. All specimens must be properly labeled and must be accompanied by the PPHSN Influenza Surveillance Form with all information completed on the form.
2. Unacceptable Specimens.
 - a. Nasopharyngeal swabs that are completely dry.
 - b. NPS specimens collected on a calcium alginate swab.
 - c. Specimens received unlabeled or mis-labeled.
 - d. Specimens received without the Influenza Surveillance Form.

E. Reagents

1. Staining Reagents.
 - a. BIORAD: MONOFLUO® KIT INFLUENZA (ref. no: 52209).
 - i. R1a: **Influenza A monoclonal mouse antibody.**
 - ii. R1b: **Influenza B monoclonal mouse antibody.**
 - iii. R2: **Negative Control.**
 - iv. R3: **Influenza A+B Conjugate – anti-mouse IgG with FITC.**
Reagent Preparation.
 - Allow reagent kit to come to room temperature.
 - Prepare conjugate by adding 9 ml PBS to vial labeled R3 (conjugate).
 - v. R4: Glycerol mounting medium.
 - b. BIORAD: MONOFLUO®SCREEN R.S.V. (ref. no: 52216).
 - i. R1: **RSV mouse monoclonal antibody with FITC conjugate.**
 - ii. R2: **Glycerol mounting medium.**
2. Other Reagents.
 - a. Phosphate Buffered Saline (PBS): Add powdered contents of vial to 1 litre of sterile distilled water, store at 2-4°C.
 - b. 100% Acetone, acetone is used at -20°C , change every 2-3 weeks, store at room temperature in appropriate safety environment.
 - c. 100% Ethanol, store at room temperature in appropriate safety environment.

F. Equipment and Supplies.

1. Fluorescent microscope.
2. Centrifuge (capable of achieving 2000 RPM).
3. Incubator (37°C).
4. Refrigerator and freezer.
5. Glass microscope slides (Immuno slides with 6-8 wells per slide).
 - a. Slides must be cleaned before use as accumulation of dust, oil or fungal growth on new slides can make reading difficult. Wash slides by gentle agitation in liquid (dishwashing) soap and rinsing with plenty of water. Rinse slides with a final rinse in distilled water. Air dry slides and make sure they are completely dry before use. Avoid using a sponge or other wiping devices as this may leave streaks on the slides.

6. Coverslips (large, 24x60mm).
7. Transfer pipets.
8. Specimen collection swabs.
9. Transport vials (screw capped, 3 ml volume).
10. Wash bottles.
11. Coplin jars (2).
12. Sterile distilled water.
13. Humid chamber slide holder (plastic box containing a wet sponge or paper towel).
14. Timer.
15. Cardboard slide holders.
16. Specimen racks.

G. Safety

1. Test procedures should be performed in a clutter free, well ventilated area.
2. Lab personnel should use lab coats and gloves and dispose of them appropriately.
3. All waste should be discarded in appropriate biohazard waste containers.
4. Flammable liquids must be used with all appropriate measures.
5. Work surfaces must be disinfected with a suitable disinfecting solution after performing all procedures. A 10% bleach solution is the recommended disinfecting solution.
6. Refer to lab safety policies and procedures prior to performing test to ensure safety measures are followed adequately.

H. Procedures

Specimen Preparation

1. Nasopharyngeal Swab Specimens (NPS).
 - a. At the time of collection NPS material is smeared onto the labeled slide, air dried and delivered to the lab. A second NPS is also collected and placed in the provided transport vial.
 - b. When received in the lab, specimens should be recorded in the lab register or accessioning system with all pertinent information.
 - c. Fix the slide by placing it in a coplin jar containing acetone, for 5 minutes, preferably at -20°C.
 - d. Remove slide from acetone and allow to air dry completely before staining.
 - e. If slide cannot be stained immediately, store at -20°C until ready to stain.
 - e. To the transport vial containing the second NPS specimen, add 1 ml of 100% ethanol, cap securely, and store at -20°C for possible further testing.
2. Nasopharyngeal Aspirate Specimens (NPA).
 - a. NPA specimens should be received in a clean, leak-proof container from the collection site.
 - b. When received in the lab, specimens should be recorded in the lab register or accessioning system with all pertinent information.
 - c. With a transfer pipet, transfer the specimen into a centrifuge tube.
 - d. Centrifuge the tube for 10 minutes at 2000 rpm.
 - e. After centrifugation is complete, remove all supernatant from the tube and transfer 1 ml into a labeled transport vial. Discard remaining supernatant in appropriate waste container.
 - f. Add 1 ml of 100% ethanol to the vial, cap securely, and store the specimen at -20°C for possible further testing.
 - g. To the remaining sediment in the centrifuge tube (step e), add 3-4 ml of PBS and resuspend by mixing gently (Wash 1).
 - h. Centrifuge the specimen for 10 minutes at 2000 rpm.
 - j. After centrifugation is complete, remove all supernatant and discard.
 - k. Add 3-4 ml of PBS to remaining sediment and resuspend by mixing gently (Wash 2).
 - l. Centrifuge the specimen for 10 minutes at 2000 rpm.
 - m. Remove the supernatant and discard.

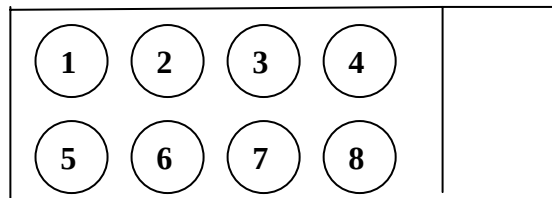
- n. Add 1 ml of PBS to the remaining sediment and resuspend by mixing gently. Suspension should appear slightly hazy. If suspension is too hazy, adjust the consistency by adding additional PBS, drop by drop until a slight haziness is achieved.
- o. To a labeled specimen slide add one drop of the final suspension to each well and allow slides to air-dry completely. Slides may be placed in a 37°C incubator to speed up drying.
- p. Fix the slide by placing it in a coplin jar containing acetone, for 5 minutes, preferably at -20°C.
- q. Remove slide from acetone and allow to air dry completely before staining.
- r. If slide cannot be stained immediately, store at -20°C until ready to stain.

Notes:

- **For NPA specimens containing large clumps of mucus: vortex the specimens vigorously and use a wood applicator stick to break-up the material. Then proceed from step 2,c above.**
- **For NPA specimens that are bloody in appearance: add 1 ml of sterile water and mix gently to lyse the red blood cells. Then proceed from step 2,c above.**

Slide Staining :

3. Bring all reagents and specimen slides to room temperature prior to staining.
4. To the specimen slide that has been acetone fixed and completely dried add: stain reagents as follows:



- a. To well #1 add 1 drop (approx. 30µl) of reagent R1a-**anti influenza A**.
- b. To well #2 add 1 drop of reagent R1b-**anti-influenza B**.
- c. To well #3 add 1 drop of reagent R2-**Negative Control**.
5. Make sure the drop covers the entire well but does not cross over into adjacent wells.
6. Place the slide in a humid chamber and incubate at 37°C for 30 minutes.
7. After 30 minutes, remove the slide from the incubator, rinse off the excess reagent by tilting slide and gently squirting the stain reagent off the slide with PBS in a wash bottle.
8. When all stain reagent is removed, place the slide in a coplin jar containing PBS.
9. Allow the slide to sit in the PBS for 5 minutes with gentle agitation to wash the slide.
10. After 5 minutes, discard the PBS, add fresh PBS and repeat steps 8 and 9.
11. After the second 5 minute wash, remove the slide and allow to dry in an upright position.
12. When slide is dry, add stain reagents as follows:
 - a. To well #1, #2, and #3 add 1 drop of reagent R3-**Influenza A+B Conjugate**.
 - b. To well #4 add 1 drop of reagent R1-**RSV antibody with FITC conjugate**.
13. Make sure the drop covers the entire well but does not cross over into adjacent wells.
14. Place the slide in a humid chamber and incubate at 37°C for 30 minutes.
15. After 30 minutes, remove the slide from the incubator, rinse off the excess reagent by tilting slide and gently squirting the stain reagent off the slide with PBS in a wash bottle.
16. When all stain reagent is removed, place the slide in a coplin jar containing PBS.
17. Allow the slide to sit in the PBS for 5 minutes with gentle agitation to wash the slide.
18. After 5 minutes, discard the PBS, add fresh PBS and repeat steps 16 and 17.
19. After the second wash, give the slide a final wash by rinsing with distilled water.
20. Allow slide to dry completely in an upright position.
21. Add a small drop of glycerol mounting medium to each well and gently press coverslip onto the slide.
22. For best results, slide should be read immediately. Slide may be read up to but not longer than 24 hours after staining, if stored at 4°C and protected from light.

I. Microscopic Slide Examination

1. Turn on the fluorescent microscope and wait for at least 15 minutes to allow fluorescent bulb to warm up (Refer to the microscope user's manual for specific operation instructions).
2. Slide should be examined using the 40X objective.
3. When examining the slide, each entire, stained well must be read.
4. Examine slides using the 40X objective.
5. For each slide, first examine the negative control well (well #3) to establish the presence of adequate clinical material and the quality of staining.
6. Read the wells in the following order: 1-Negative control well; 2-Flu A stained well; 3-Flu B stained well; 4-RSV stained well.
7. Prepare a worksheet for every batch of slides to be examined and record the results on the worksheet (see attachment 2). The following information should be included on the worksheet (an example worksheet is included in the attachments).
 - a. Patient name and ID number.
 - b. Date slides are stained and initial of tech performing the stain.
 - c. Date slides are read, performing technician and results of slide examinations.
 - i. Record positive results as: Positive: Influenza A; Positive: Influenza B; Positive: RSV.
 - ii. Positive results must also include a score (see interpretation criteria below).
 - iii. Record negative results as: Negative.
 - iv. Record poor quality specimens as: Inadequate.

J. Interpretation of Slide Examination

1. Positive Result:
 - a. Presence of cells displaying bright, apple-green fluorescence .
 - b. Staining should be seen in components within the cellular structure, however, in strong infections, all cell structures will fluoresce giving the appearance of one whole fluorescent cell.
 - c. With RSV positive staining, the intensity of staining will show in the cell structure and also in areas surrounding the cells.
 - d. Score all positive results using the following criteria:
 - i. 1+: Positive cells seen in $\leq 10\%$ of entire well.
 - ii. 2+: Positive cells seen in 10%-50% of entire well.
 - iii. 3+: Positive cells seen in 50%-100% of entire well.
2. Negative Result:
 - a. Absence of cells displaying bright apple-green fluorescence.
 - b. Cells will appear red with no characteristic fluorescence within the cell structure.
 - c. Non-specific staining: occasionally, the cells may demonstrate a staining appearance that may be confused as fluorescence.
 - i. This will be a yellow to light green appearance and will be diffused through the whole cell structure and will also be seen in most of the cell monolayer in each well.
 - ii. In most cases this non-specific staining is due to specimen quality, and/or problems in staining and washing techniques.
 - iii. It is essential to first examine the Negative control well (well #3) for each slide and use this as a reference to compare with all other stained wells of the same slide.
3. Quality of Specimen
 - a. For a valid result: upon examination, 10 epithelial cells must be present in each stained well (wells 1, 2, 3, and 4).
 - b. The exception to this requirement is if one of the stained wells demonstrates a characteristic positive result and less than 10 epithelial cells was observed in all cells.

4. Possible Problematic Staining and Explanations

	Problem Observed	Possible Explanation
1.	Poor fluorescence observed	Microscope not allowed 15 minutes to warm-up
2.	Fluorescence appears uneven and hazy	Slides not at room temperature before Reading
3.	Strong fluorescence (specific and non-specific) in all wells	Inadequate washing performed during stain process
4.	For QC specimen, all wells are negative	Reagents not added or added incorrectly during staining process
5.	Unable to focus on wells	- Slide stained on the wrong side - Coverslip not placed on wells or not placed on the correct side

K. Reporting:

1. Record all slide results on the designated lab worksheet.
2. From the worksheet, prepare the patient report information.
3. On the Influenza Surveillance Form (see attachment) fill in the following information
 - a. In the Lab investigation section, check the box indicating the test performed.
 - b. In the Lab conclusion section, circle all results determined from the slide examination (there may be very rare instances where more than 1 positive result is seen from the same specimen).

L. Quality Control

1. QC slides should be stained and read alongside patient samples once a week or when a new shipment of stain reagents is received.
2. Record the results of QC slides on the QC recording form (see attachment 3).
3. Acceptable QC results are:

Influenza A Control	Presence of Characteristic Apple-Green Fluorescence of 1+ or greater
Influenza B Control	Presence of Characteristic Apple-Green Fluorescence of 1+ or greater
RSV Control	Presence of Characteristic Apple-Green Fluorescence of 1+ or greater
Negative Control	Absence of Characteristic Apple-Green Fluorescence

4. If QC results do not meet the above criteria, the following steps should be followed to determine if patient results can be reported.
 - a. Check to see if stains/reagents are still within expiration dates.
 - b. Check to see if stains/ reagents have been stored and handled according to manufacturer's instructions.
 - c. Review staining procedure to see if all steps were followed appropriately.
 - d. Check if the slide was stained on the side with specimen and that specimen material was not removed prior to staining.
 - e. Repeat the staining procedure on a new QC slide.
 - f. If results meet the QC criteria, patient results may be reported.
 - g. If results still do not meet the QC criteria, do not report patient result, consult lab supervisor for assistance.

M. Limitations:

1. It is important that slides are pre-cleaned prior to use as dirt on the slides may absorb the fluorescent stain resulting in false positive result interpretations.

2. Stain reagents should always be stored at refrigerated temperatures and care should be taken in handling the reagents so as to minimize bacterial contamination. Contaminated stains may alter the performance of the test and may also result in false positive interpretations.
3. It is important to assess the quality of each specimen and to report those specimens not meeting the requirement as Inadequate and not as a negative result.
4. It is important that clinicians and other health care workers involved in collecting specimens are fully familiar with the collection criteria required.
5. For limitations on stain procedures please refer to the BioRad package inserts.

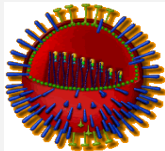
N. References

1. Biorad MONOFLUO®KIT INFLUENZA and MONOFLUO®SCREEN RSV package inserts.
2. Pasteur Institute of New Caledonia "Detection of respiratory virus by immunofluorescence" procedure manual.

O. ATTACHMENTS

1. Influenza Surveillance Form (IPD-4)
2. Microscopy Result Worksheet (IPD-8)
3. QC Recording Form (IPD-9)

ATTACHMENT 1

	- PPHSN - Influenza Surveillance Network Transmission form for influenza testing	
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Clinician/Health Worker :

Name:

Signature: Date:

Clinic/Hospital Site:

Patient Id :

Last Name:

First Name:

D.O.B.:/...../..... (d/m/y) Sex : F ☐ M ☐.

Current

Address :

Epidemiological Context :

Sporadic case: Yes ☐ No ☐ Familial outbreak: Yes ☐ No ☐ Community outbreak: Yes ☐ No ☐

Other (specify):

Vaccinated against Flu ☐ Date:/...../.....(d/m/y) Not Vaccinated ☐

Recent travel: Yes ☐ Specify (where – when):
No ☐

Clinical features :

First day of illness with ILI:/...../.....(d/m/y)

Influenza-Like Illness (ILI) Case Definition:

Sudden onset of fever >38°C Yes ☐ No ☐

Cough Yes ☐ No ☐

Sore throat Yes ☐ No ☐

Myalgia Yes ☐ No ☐

Frequently associated symptoms: headache, tiredness, runny nose
(circle all symptoms observed)

Other symptoms (specify):

Biological samples collected (or prescribed):

Sample date :/...../..... (d/m/y)

Sample Collected: NasoPharyngeal swab ☐ NasoPharyngeal aspirate ☐

Other (specify):

To be completed by the laboratory and then transmitted to local MoH/DoH

Lab investigation: IFA direct examination ☐ Other test:

Lab conclusion (circle result): NEGATIVE FLU A FLU B RSV INADEQUATE PECIMEN

Referred Specimen (circle one): Ethanol Preserved Frozen

Name of Reference Lab: Date Shipped:

Signature and Date:

Reference Lab Conclusion (circle result): NEGATIVE FLU A FLU

ATTACHMENT 2
INFLUENZA FLUORESCENT STAIN RESULT WORKSHEET

Date Slides Stained		Stained By	
Date Slides Read		Read By	

#	Patient Name	Patient ID #	Exam Result
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			

ATTACHMENT 3
INFLUENZA FLUORESCENT STAIN QUALITY CONTROL RECORD

Instructions:

1. A control slide must be stained once a week or when a new shipment of stain reagent is received.
2. Control slides must be stained in the same manner that patient slides are stained (see stain procedure).
3. All QC slides may not have the same viruses on each slide. Record the results observed for each slide.
4. Acceptable Performance for Quality Controls are

Influenza A Control	Presence of Characteristic Apple-Green Fluorescence of 1+ or greater
Influenza B Control	Presence of Characteristic Apple-Green Fluorescence of 1+ or greater
RSV Control	Presence of Characteristic Apple-Green Fluorescence of 1+ or greater
Negative Control	Absence of Characteristic Apple-Green Fluorescence

5. Record the observed QC results in the record chart below
6. If the QC does not perform as described above, do not report results, refer to QC troubleshooting steps in the staining procedure.
7. Fill in the Lot# and expiration date information below

	Test Component	Lot#	Expiration Date
1	BioRad Monofluo Influenza Stain Kit		
2	BioRad Monofluo RSV Stain Kit		

	Influenza A Control	Influenza B Control	RSV Control	Negative Control	Date Read	Initial
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
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18						